

Targeting of Adenovirus E1A and E4-ORF3 Proteins to Nuclear Matrix-associated PML Bodies

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Abstract. The PML protein was first identified as part of a fusion product with the retinoic acid receptor α (RAR α), resulting from the t(15;17) chromosomal translocation associated with acute promyelocytic leukemia (APL). It has been previously demonstrated that PML, which is tightly bound to the nuclear matrix, concentrates in discrete subnuclear compartments that are disorganized in APL cells due to the expression of the PML-RAR α hybrid. Here we report that adenovirus infection causes a drastic redistribution of PML from spherical nuclear bodies into fibrous structures. The product encoded by adenovirus E4-ORF3 is shown to be responsible for this reorganization and to colocalize with PML into these fibers. In addition, we demon-

strate that E1A oncoproteins concentrate in the PML domains, both in infected and transiently transfected cells, and that this association requires the conserved amino acid motif (D)LXCXE, common to all viral oncoproteins that bind pRB or the related p107 and p130 proteins. The SV-40 large T antigen, another member of this oncoprotein family is also found in close association with the PML nuclear bodies. Taken together, the present data indicate that the subnuclear domains containing PML represent a preferential target for DNA tumor viruses, and therefore suggest a more general involvement of the PML nuclear bodies in oncogenic processes.

THE eukaryotic nucleus is highly organized into discrete domains which spatially separate different biochemical processes. A variety of metabolic activities such as DNA replication, ribosome assembly, transcription, and pre-mRNA processing localize to distinct subnuclear compartments. The most conspicuous example is the nucleolus in which rRNAs are assembled into ribosomal subunits (reviewed by Scheer and Weisenberger, 1994). DNA replication sites were also shown to concentrate within discrete regions containing the proliferating cell nuclear antigen (PCNA;¹ Bravo and Macdonald-Bravo, 1987) as well as DNA methyltransferase (Leonhardt et al., 1992). In addition, the small nuclear ribonucleoprotein particles (snRNPs), which are the major subunits of spliceosomes, were similarly found to localize to

distinct subnuclear structures corresponding to interchromatin granules, perichromatin fibrils, and coiled bodies (reviewed in Lamond and Carmo-Fonseca, 1993).

The systematic characterization of autoimmune antibodies that selectively label individual subcellular domains has recently identified a novel type of nuclear structures. These structures, defined previously by electron microscopists as a sub-type of nuclear bodies (reviewed in Bouteille et al., 1974), appear as dense spherical particles, 0.3–0.5 μ m in diameter, that often display a doughnut-like morphology. To date, at least four different proteins have been shown to be components of these nuclear bodies: a 65-kD protein (Epstein, 1984), NDP55 (Ascoli and Maul, 1991), SP100 (Szostecki et al., 1990; Xie et al., 1993), and PML (Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994). The cDNAs encoding two of these proteins have been isolated (SP100 and PML). The amino acid sequence of SP100 did not reveal any significant homology with known proteins, while PML appeared as a new member of a growing family of proteins characterized by the presence of a Cys/His-rich cluster referred to as a RING finger (Freemont et al., 1991), one or two additional Cys/His-rich motifs (B boxes), and a predicted coiled-coil region. No apparent functional similarity exists between the seven

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1. *Abbreviations used in this paper:* Ad2, adenovirus serotype 2; APL, acute promyelocytic leukemia; BrdU, bromodeoxyuridine; f.f.u., focus forming units; hsp70, 70-kD heat shock protein; HSV, Herpes Simplex Virus; LT, large T antigen; ORF, open reading frame; PBS-Tw, PBS containing 0.05% Tween-20; PCNA, proliferating cell nuclear antigen; RA, retinoic acid; RAR α , retinoic acid receptor α ; snRNP, small ribonucleoprotein particles.

members of the family described so far (Reddy et al., 1992).

PML was first identified as part of a fusion product with the retinoic acid receptor α (RAR α), resulting from the t(15;17) chromosomal translocation associated with acute promyelocytic leukemia (APL) (de Thé et al., 1991; Kikuzuka et al., 1991; Pandolfi et al., 1991; Goddard et al., 1991; Kastner et al., 1992). This leukemia is characterized by a block in differentiation at the promyelocyte stage and a particular sensitivity to retinoic acid (RA) (Huang et al., 1988; Castaigne et al., 1990). We and others have shown that the PML-RAR α hybrid displays an altered nuclear localization when compared to that of its normal PML and RAR α counterparts (Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994). In APL cells, most, if not all of the PML nuclear bodies are disorganized into numerous and aberrant microstructures containing both PML and PML-RAR α . Strikingly, RA treatment induces a drastic reorganization of the altered PML nuclear bodies back to their normal number and morphology, correlating with the therapeutic effect of RA in the disease. These data strongly suggest that the disruption of the PML subnuclear structures may play an important role in APL pathogenesis.

Adenovirus serotype 2 (Ad2) is a DNA tumor virus that infects permissive human cells in a lytic cycle. In addition, most adenoviruses have transforming properties in rodent cells and some serotypes can induce tumors when injected into newborn hamsters or rats (reviewed by Boulanger and Blair, 1991; Bayley and Mymryk, 1994). The minimal sequences required for adenovirus transformation encompass the early region 1A (E1A) and E1B genes. The two major E1A proteins, which are translated from the 13S and the 12S mRNAs, play an essential role in both stimulating viral gene transcription and inducing extended proliferation of rodent cells (reviewed by Akusjärvi, 1993; Bayley and Mymryk, 1994). However, the full transformed phenotype requires the coexpression of E1B. The E1A gene encodes three regions that are well conserved between different serotypes and that correspond to three independent functional domains (designated CR1, CR2, and CR3; see Fig. 8) (Kimelman et al., 1985; Moran and Mathews, 1987). The CR3 region, that is required for efficient transactivation, is not essential for transformation (Lillie et al., 1987; Schneider et al., 1987). In contrast, the CR1 and the CR2 regions together with the extreme NH₂ terminus are essential for transformation- and cell growth-regulating activities of E1A (reviewed in Moran, 1993). These noncontiguous sequences are critical for the binding of cellular proteins such as the retinoblastoma (pRB) gene product, the pRB-related p107 and p130 proteins and the p300 cellular product (Yee and Branton, 1985; Harlow et al., 1986; Whyte et al., 1988; Ewen et al., 1991; Eckner et al., 1994).

A number of different proteins are likely encoded by the adenovirus early region 4 (E4), corresponding to seven different translational open reading frames (ORFs) (Virtanen et al., 1984; Freyer et al., 1984) (see Fig. 4). Two of them, ORF3 and ORF6, have redundant effects in viral lytic infection (Huang and Hearing, 1989; Bridge and Ketner, 1989). While mutation in either of the two proteins essentially results in a normal productive infection, a double-mutant, defective in both ORF3 and ORF6 expression,

grows extremely poorly and shows multiple lesions including defects in viral DNA replication, late viral mRNA accumulation and protein synthesis as well as failure in the shut-off of host cell protein synthesis (Halbert et al., 1985; Weinberg and Ketner, 1986; Huang and Hearing, 1989; Bridge and Ketner, 1989). Little is known concerning the biological activities of the ORF3 and ORF6 proteins. However, recent studies have shown that they exhibit some features characteristic of RNA splicing factors (Öhman et al., 1993). The ORF3 product functions as an exon inclusion factor whereas the ORF6 product preferentially favors exon skipping (Nordqvist et al., 1994).

Viruses require the host cell machinery for their life cycles, and viral infection provides an amenable model system for the functional study of host cell subnuclear structures. Since it was previously shown that a type of nuclear structure, the PML nuclear bodies, is disorganized during APL pathogenesis, we wished to investigate the effects of small DNA tumor virus infection on the integrity of these structures. Here we show that adenovirus infection causes a dramatic redistribution of PML from doughnut-shaped nuclear bodies into fibrous structures and that the E4-ORF3 protein is both necessary and sufficient for this redistribution. The E1A oncoproteins are found to colocalize with PML, both in the intact and filamentous PML structures and the conserved region 2 appears to be critical for this association. The SV-40 large T protein is also shown to accumulate in close proximity to the PML nuclear bodies. These results suggest that the PML subnuclear domains represent specialized nuclear matrix regions that are preferential targets for small DNA tumor virus oncoproteins.

Materials and Methods

Cell Culture, Adenovirus Infection, and Transfection Assays

HeLa cells were grown in Dulbecco's modified minimum essential medium, supplemented with antibiotics, glutamine and 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD). Subconfluent cells were infected with adenoviruses subtypes 2 and 5 at a multiplicity of infection of 20–50 focus forming units (f.f.u.) per cell, as previously described (Pombo et al., 1994).

Transfections were performed by the calcium phosphate precipitation method. HeLa cells were grown as monolayers on glass coverslips in 35 mm Petri dishes containing 1 ml of culture medium and transfected with 4–5 μ g of expression vector DNA. 24 h after transfection the medium was replaced by fresh culture medium and the cells were further incubated for 24–48 h. All transfections were independently repeated at least three times.

Immunofluorescence

Cells grown on coverslips were washed twice in PBS and fixed and permeabilized according to one of the following protocols: (a) Triton post-fixation. Cells were fixed in 3.7% paraformaldehyde (PFA) in PBS for 10 min at room temperature, and then permeabilized with 0.5% Triton X-100 in PBS, for 20 min at room temperature. (b) Triton pre-fixation: cells were extracted with 0.5% Triton X-100 in CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 2 mM EDTA) containing 0.1 mM PMSF, for 1–3 min on ice, and then fixed in 3.7% PFA in CSK buffer, for 10 min at room temperature, according to Fey et al. (1986); (c) SDS post-fixation: cells were fixed in 3.7% PFA in PBS for 10 min at room temperature, and then permeabilized with 0.2% SDS in 20 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 30 mM NaCl, for 20 min at room temperature.

After fixation and permeabilization, the cells were rinsed in PBS containing 0.05% Tween-20 (PBS-Tw), incubated with primary antibodies for

1 h, washed in PBS-Tw, and further incubated for 1 h with the appropriate secondary antibodies conjugated with either fluorescein or Texas red (Dianova GmbH, Hamburg, Germany). For double-labeling experiments, incubation with the primary antibodies was performed sequentially. Finally, the samples were mounted in VectaShield (Vector Laboratories, Burlingame, CA). The following primary antibodies were used: anti-PML rabbit polyclonal serum (Weis et al., 1994), anti-PML mAb 5E10 (Stuurman et al., 1992), human patient serum Kr (Weis et al., 1994), anti-SP100 rabbit polyclonal serum (Xie et al., 1993), anti-Sm mAb Y12 (Lerner et al., 1981), anti-E1A mAbs M1 and M73 (Harlow et al., 1985), anti-SV-40 LT mAb Ab-1 (Oncogene Science, Inc., Mineola, NY), anti-E4-ORF3 protein mAb A4-7, anti-E4-ORF6 protein mAb M45 (Obert et al., 1994), anti-pRB mAb G3-245 (Hu et al., 1991), anti-p300 mAbs RW128 and RW105 (a gift from Dr. R. Eckner, Dana-Farber Cancer Institute, Boston, MA), and anti-hsp70 mAb SPA-810 (StressGen, Victoria, B.C., Canada).

Visualization of Replication Sites

To visualize the sites of viral DNA replication, cells were infected for 8–10 h, and then pulse-labeled *in vivo* with 150 μ M bromodeoxyuridine (BrdU; Boehringer Mannheim GmbH, Mannheim, Germany) for 20 min. Detection of incorporated BrdU was performed as previously described (Pombo et al., 1994). Aphidicolin (5 μ g/ml) was present in the culture medium for 14 h prior to the *in vivo* pulse with BrdU in order to inhibit the cellular DNA polymerase α . Control experiments demonstrated that treatment with aphidicolin under these conditions abolishes incorporation of BrdU into uninfected cells but does not affect detection of viral replication (Pombo et al., 1994).

Microscopy

Samples were examined with a Zeiss Axiophot fluorescence microscope. Images were recorded using either a Hamamatsu SIT camera and an ARGUS 10 image processor (Hamamatsu Photonics) or the confocal microscope. Confocal microscopy was performed with the Zeiss laser scanning microscope LSM 310, using excitation wavelengths of 488 nm (for fluorescein) and 543 nm (for Texas red). The two channels were recorded independently and pseudocolour images were generated and superimposed. Images were photographed on Kodak TMAX 100 film, or directly printed using a Kodak XLS 8300 Digital Printer. Immuno-electron microscopy was performed on both non-infected and adenovirus-infected HeLa cells as described previously (Weis et al., 1994).

Plasmid DNA

Plasmid pE1A contains nucleotides 1 to 1,773 of genomic Ad2 sequences (pML 005 in Bondesson et al., 1994). Variants of this parental plasmid were constructed. The suffixes 12S and 13S denote that genomic E1A sequences were replaced by cDNA sequences taken from the E1A-12S and E1A-13S mRNAs (Perricaudet et al., 1979). Δ CR1, Δ CR2a, and Δ CR2b are in-frame deletion mutants lacking amino acids 38–65, 121–125, and 125–133, respectively (Schneider et al., 1987). Variant Δ CR1,2a was constructed by combining the deletions in the Δ CR1 and Δ CR2a mutants. The p12S. Δ COOH terminus mutant was derived from pKGO-007 Doff (Linder et al., 1991) and lacks the amino acids 140–243. Plasmids p12S.YH47, p12S.pm928, p12S.YH47/pm928, and p12S.RG2 were kind gifts from Elizabeth Moran (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and have been described previously (Wang et al., 1993). CMV expression plasmids encoding E4-ORF3, ORF6, ORF6/7 are described in Öhman et al. (1993). The pDept vector expressing the SV-40 LT antigen was described in Banerji et al. (1983). The pCMVRB expression vector was a gift from Kristian Helin (Danish Cancer Society, Copenhagen).

Western Blot Analysis

HeLa cells were infected with mutant virus H5d1366-ORF3 (making E4-ORF3; Huang and Hearing, 1989a) or H5ilE41 (making E4-ORF6 and E4-ORF6/7; Hemström et al., 1988) as described by Nordqvist et al. (1994). 293 cells were transfected with plasmids pUC19, pCMVE4, pCMVE4-ORF3, pCMVE4-ORF6, or pE4-ORF3⁻ (Öhman et al., 1993). Cells were harvested 28-h postinfection and 48-h posttransfection, lysed by treatment with IsoB-0.5% NP-40, and fractionated into nuclei and cytoplasm (Nordqvist et al., 1994). Nuclei were disrupted with Laemmli sample buffer and total nuclear proteins separated by electrophoresis in a 15% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto a nitrocellulose

membrane. Expression of the E4-ORF3 protein was detected using the ECL system as described by the manufacturer (Amersham). The monoclonal antibody A4-7 was prepared against the E4-ORF3 protein produced in *Escherichia coli* as a histidine fusion protein (Novagen, Inc., Madison, WI).

Results

Adenovirus Infection Causes a Redistribution of PML

Immunofluorescence analysis of uninfected HeLa cells using an anti-PML serum reveals a variable number of bright dots in the nucleus (Fig. 1 A), as previously described (Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994). In contrast, at 6–8 h postinfection with adenovirus, the anti-PML serum labels a meshwork of fibrous structures in the nucleoplasm (Fig. 1 B), which appear to remain unaltered up to 24 h after infection. Such fibers were visible in every infected cell as checked by double labeling with an antibody directed against the adenovirus DNA-binding protein and the PML antiserum (data not shown). Similar results were obtained in MCF-7 cells infected with either

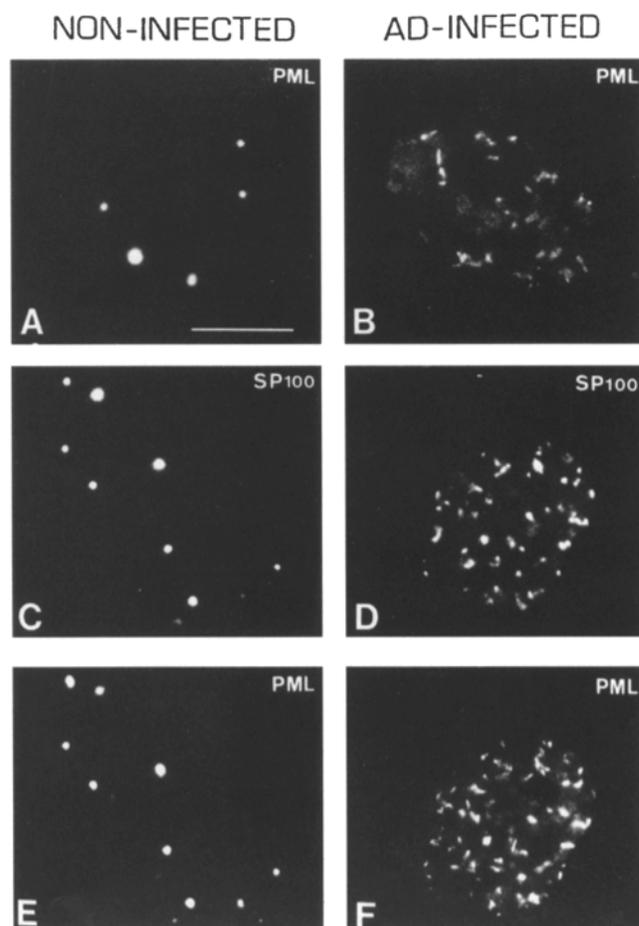


Figure 1. Adenovirus infection redistributes PML. Indirect immunofluorescence was performed using anti-PML serum on HeLa cells before (A) and after Ad2 infection (B, 8-h postinfection). The cells were fixed with paraformaldehyde and permeabilized with Triton X-100. The distribution of PML was compared to that of SP100 in the nucleus of non-infected (C and E) and infected (D and F) HeLa cells using human autoimmune Kr (C and D), and rabbit anti-PML (E and F) sera. Bar, 10 μ m.

adenovirus type 2 or 5 and labeled with either rabbit anti-PML serum or anti-PML monoclonal antibody 5E10 (Figs. 1 and 4; and data not shown).

Since the nuclear bodies that contain PML represent a macromolecular multiprotein complex (Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994), it was important to

determine whether adenovirus acts exclusively on PML or whether it affects the whole complex. Therefore, we performed immunofluorescence of infected HeLa cells using antibodies specific for another component of the PML domains, the SP100 protein (Szostecki et al., 1990; Xie et al., 1993). We tested both polyclonal rabbit antisera raised

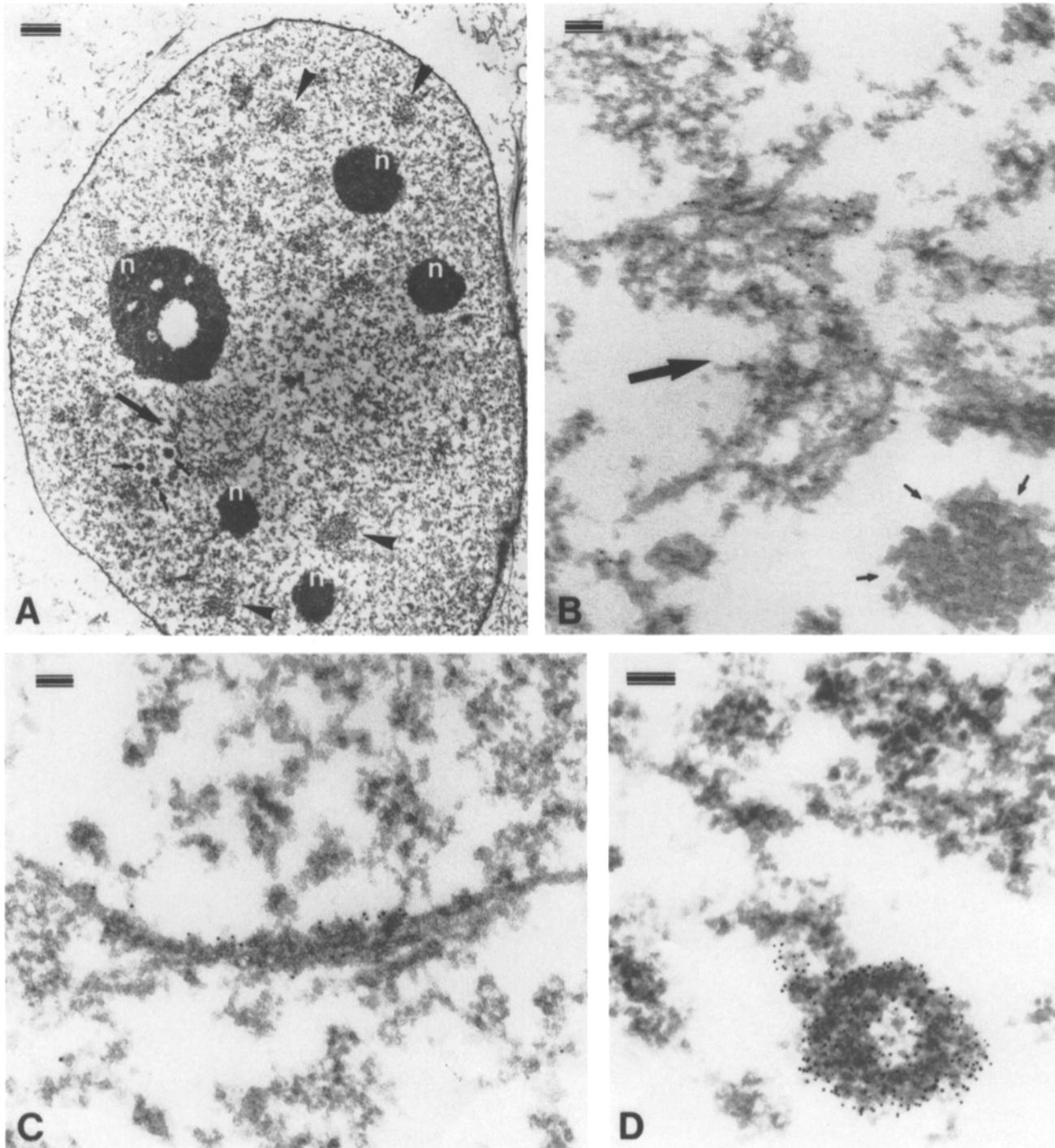


Figure 2. Localization of PML by electron microscopy. *A–C* depict the nucleus of Ad2 infected cells at 9-h postinfection. The nucleoplasm contains prominent clusters of interchromatin granules (*A*, arrowheads) and several viral-induced fibrillar spheres (*A* and *B*, small arrows). The nucleoli appear compact and predominantly granular (*A*, *n*). In addition, nuclear filaments are frequently observed (*A* and *B*, arrow; the area indicated in *A* by the large arrow is shown at higher magnification in *B*). The filaments form meshworks (*B*) or bundles (*C*) and are labeled by PML antibodies. For comparison, *D* depicts a typical doughnut-shaped structure labeled by PML antibodies in non-infected cells. Bars: (*A*) 1 μm ; (*B–D*) 0.1 μm .

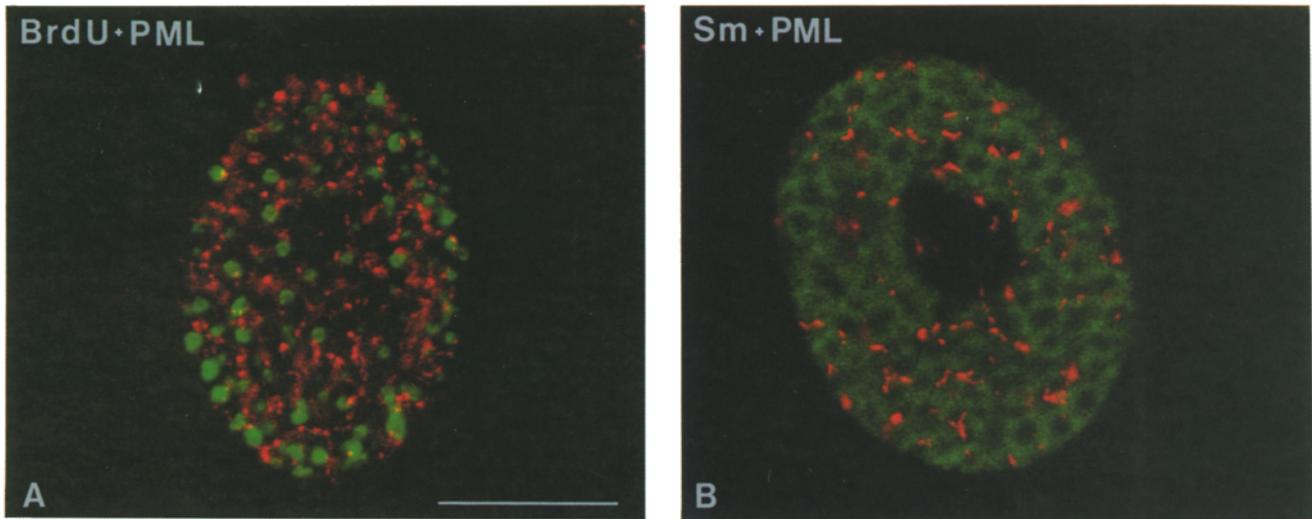


Figure 3. The PML fibrous structures do not correspond to sites of viral DNA replication or RNA processing. (A) HeLa cells were infected with Ad2 for 8 h incubated for 30 min with bromodeoxyuridine (*BrdU*) and labeled with PML antibodies. The sites of replication were visualized using anti-*BrdU* antibodies coupled to fluorescein (*green* staining) and PML was detected with antibodies coupled to Texas red (*red* staining). Confocal images from each fluorochrome were taken separately and superimposed. (B) HeLa cells infected for 16 h were incubated with mAb Y12, which reacts with common proteins in each of the splicing snRNP (*green* staining) and PML antibodies (*red* staining). Bar, 10 μ m.

against recombinant SP100 (Szostecki et al., 1990; Xie et al., 1993), and the Kr human autoantiserum which recognizes the same antigen (Weis et al., 1994). Surprisingly, all of these antibodies failed to react with the majority of infected cells, contrasting with a bright staining of uninfected cells (Fig. 1 C; and data not shown). However, in a small percentage of infected cells the SP100 antigen was observed in association with the fibrous structures containing PML (Fig. 1, D and F).

To further characterize the viral-induced structures that contain PML, we used PML antibodies to perform immunoelectron microscopy. At 9–12 h after adenovirus infection, the PML antibodies label meshworks or bundles of nuclear filaments of \sim 12 nm in diameter (Fig. 2, A–C). This contrasts markedly with the larger, \sim 0.5- μ m-diam round PML-containing nuclear bodies present in non-infected cells (Fig. 2 D; see also Weis et al., 1994; Koken et al., 1994).

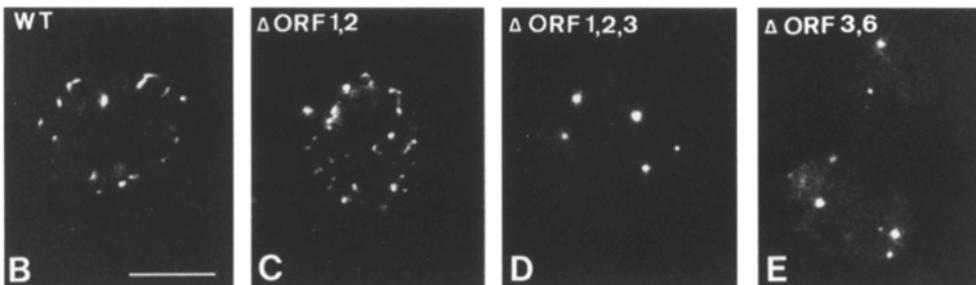
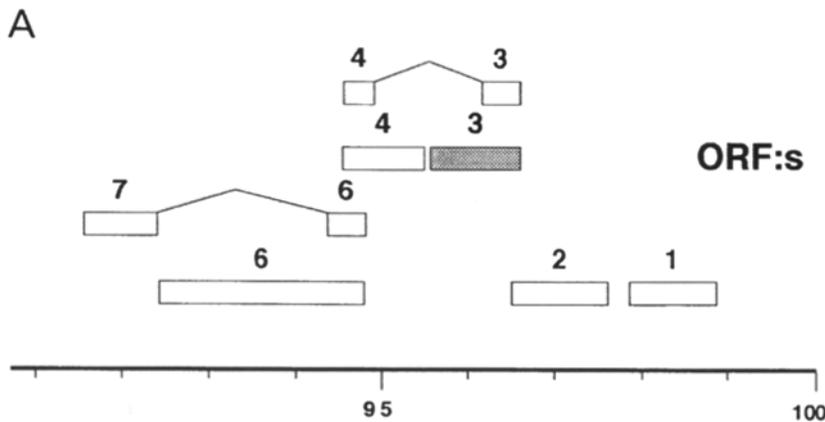


Figure 4. Mutations in the E4 gene abrogate the viral-induced redistribution of PML. (A) Schematic representation of proteins (*ORFs*) encoded by the Ad E4 transcription unit. The position of E4-ORF3, which is most relevant for this study, is shown shaded. (B–E) HeLa cells were infected with either wild-type virus Ad5 (B) or the following Ad5 mutants: H5dl 1005, which deletes ORFs 1 and 2 (C); H5dl 1006, which deletes ORFs 1, 2 and 3 (D); and H5dl 1007, which deletes ORFs 3 and also affects the ORF6 product (E). At 12-h postinfection, the cells were analyzed by immunofluorescence using PML antibodies. Bar, 10 μ m.

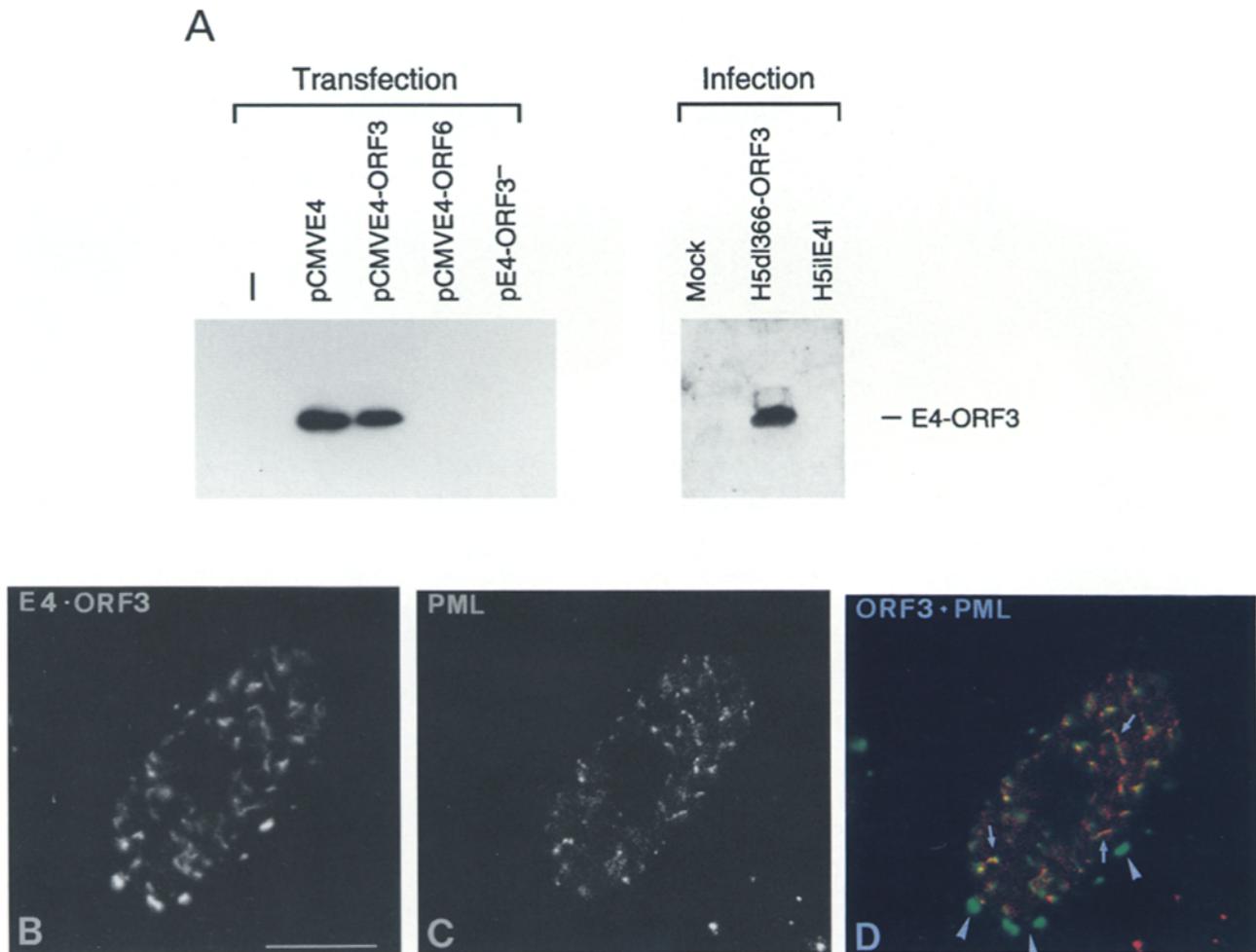


Figure 5. The E4-ORF3 gene product colocalizes with PML in infected cells. (A) Western blot analysis: nuclear extracts from 293 cells transfected with the indicated expression plasmids (lanes 1–5, from left), and nuclear extracts from uninfected HeLa cells (lane 6), and from HeLa cells infected with H5dl366-ORF3 (encoding E4-ORF3, lane 7) and H5ilE4I (encoding E4-ORF6 and ORF6/7, but not ORF3, lane 8) were electrophoresed and immunoblotted with A4-7, a mAb raised against bacterially-produced E4-ORF3 protein. Specific E4-ORF3 band is indicated. (B–D) HeLa cells infected for 8 h were fixed with paraformaldehyde, incubated with 0.2% SDS and double-labeled using mAb A4-7 and PML antibodies. The binding sites of mAb A4-7 were visualized with fluorescein (B) and those of PML antibodies with Texas red (C). Confocal images from each fluorochrome were recorded and superimposed (D; green staining for the E4-ORF3 protein, red staining for PML). Overlapping of red and green staining produces a yellowish color (arrows). Note the presence of cytoplasmic structures labeled by the mAb A4-7, which are not labeled by PML antibodies (arrowheads). Bar, 10 µm.

We next wished to determine whether the viral-induced fibrous structures containing PML correspond to sites of viral replication, transcription or RNA processing. To visualize the sites of viral replication, infected cells were incubated with BrdU in the presence of aphidicolin in order to specifically inhibit the cellular DNA polymerase α (Pombo et al., 1994). Double-labeling experiments using BrdU and PML antibodies showed no colocalization between the PML fibers and the sites of replication (Fig. 3 A). In addition, these experiments revealed the presence of fibrous structures stained by PML antibodies in cells that did not incorporate BrdU, indicating that these structures form before the onset of viral replication. We also compared the staining pattern of PML in infected cells with the labeling produced by Sm antibodies, which react with common proteins in each of the splicing snRNPs (Lerner et al., 1981). The labeling pattern of Sm antibodies has been previously shown to colocalize with the sites of

viral transcription in the nucleus of infected cells and thus presumably represents the sites of viral RNA splicing (Jiménez-García and Spector, 1993; Pombo et al., 1994). Confocal overlays of the separate PML and snRNP labeling patterns show that they do not colocalize (Fig. 3 B). We therefore conclude that the PML fibrous structures are distinct from sites of viral DNA replication, transcription, and RNA processing.

The E4-ORF3 Gene Product Is Responsible for PML Redistribution

To identify which viral protein(s) cause(s) the redistribution of PML, HeLa cells were infected with a variety of adenovirus mutants and tested for the pattern of PML distribution. We observed that in cells infected with some types of E4 mutants, the normal dot-like distribution of PML remained unaltered (Fig. 4). Since the E4 region is a very

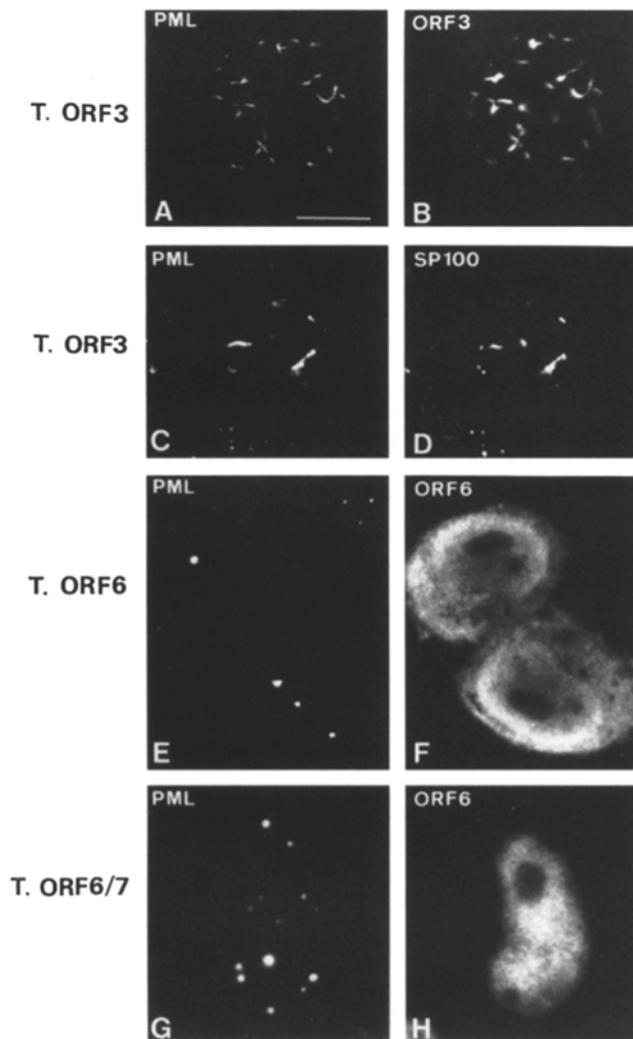


Figure 6. Expression of E4-ORF3 is sufficient to redistribute PML. HeLa cells were transiently transfected with CMV expression vectors encoding either E4-ORF3 (A–D), E4-ORF6 (E and F) or E4-ORF6/7 (G and H). The cells were analyzed by immunofluorescence using antibodies directed against PML (A, C, E, and G), E4-ORF3 protein (B), SP100 (D), and E4-ORF6 protein (F and H). Bar, 10 μ m.

complex transcription unit that encodes a minimum of seven proteins (Fig. 4 A) (Freyer et al., 1984; Virtanen et al., 1984), we made use of distinct deletion mutations of E4 in order to identify the protein(s) required for PML redistribution. The following E4 mutations were tested (Bridge and Ketner, 1989): (a) H5dl 1005, which deletes ORFs 1 and 2; (b) H5dl 1006, which deletes ORFs 1, 2, and 3; and (c) H5dl 1007, which deletes ORF3 but also affects the ORF6 product. The results show that, whereas mutant dl 1005 induces the typical filamentous structures with an efficiency similar to that of the wild-type virus (Fig. 4 C), E4 mutants dl 1006 and dl 1007 fail to alter PML distribution (Fig. 4, D and E). Therefore, the protein encoded by E4-ORF3 appears to be responsible for PML redistribution.

The intracellular localization of the adenovirus E4-ORF3 protein was analyzed using a monoclonal antibody (A4-7) generated against the E4-ORF3 gene product.

Western blot analysis indicates that this antibody reacts specifically with the viral 11-kD protein encoded by E4-ORF3 both in E4 transfected cells and during lytic virus infection (Fig. 5 A). Indirect immunofluorescence performed in infected cells revealed that mAb A4-7 stains a reticular meshwork in the nucleoplasm (Fig. 5 B) and double-labeling experiments show that both ORF3 and PML antibodies label the same fibrous structures (Fig. 5, B–D). Finally, transient DNA transfection experiments using CMV expression vectors encoding single E4-ORFs demonstrated that expression of ORF3 is sufficient to induce the formation of a meshwork of intranuclear filamentous structures labeled by antibodies directed to ORF3 and PML (Fig. 6, A and B). Similarly to what we observed in infected cells, these structures were stained by the SP100 antiserum (Fig. 6, C and D).

In contrast to the effect of E4-ORF3, overexpression of E4-ORF6 in transfected cells does not alter the normal PML distribution and, in these cells, an antibody specific for the E4-ORF6 protein produces a diffuse staining both in the nucleus and in the cytoplasm (Fig. 6, E and F). This finding is important since previous studies have shown that the ORF3 and ORF6 products appear to have redundant activities in supporting lytic virus growth (Huang and Hearing, 1989; Bridge and Ketner, 1989). In addition, transfection of an E4-ORF6/7 expression plasmid does not alter the normal PML distribution and the viral hybrid protein is detected diffusely distributed throughout the nucleoplasm (Fig. 6, G and H).

The Adenovirus E1A Oncoproteins Colocalize with PML in the Nucleus of Virus-infected Cells

Having established that adenovirus infection causes a redistribution of PML into fibrous structures, we next sought to investigate whether the E1A oncoproteins may associate with these virus-induced structures. We carried out double-labeling experiments on infected HeLa cells using PML serum and monoclonal antibody M73 raised against E1A (Harlow et al., 1985). When cells were first fixed with paraformaldehyde and then extracted with Triton X-100, mAb M73 produced widespread staining throughout the nucleoplasm (Fig. 7 I). However, when the cells were extracted with Triton X-100 prior to fixation with paraformaldehyde, this diffuse staining was significantly reduced and a meshwork of nucleoplasmic fibers became visible (Fig. 7 A). Such extraction *in situ*, while removing the soluble proteins, leaves the nuclei intact (Mittnacht and Weinberg, 1991; Fey et al., 1986). These fibrous structures were labeled by both mAb M73 and the PML serum (Fig. 7, A–C), demonstrating that the E1A proteins localize to the PML domains in the nucleus of infected cells.

A reticular pattern of E1A had been described previously in the nuclei of infected cells and shown to colocalize with the 70-kD heat shock protein (hsp70) (White et al., 1988). To test whether hsp70 may concentrate within the virus-induced PML fibers, we double-labeled infected cells with the PML serum and anti-hsp70 mAb SPA-810. In a small percentage of cells, we found a clear overlay between both staining patterns (data not shown), indicating that hsp70 colocalizes with E1A in the PML filamentous structures.

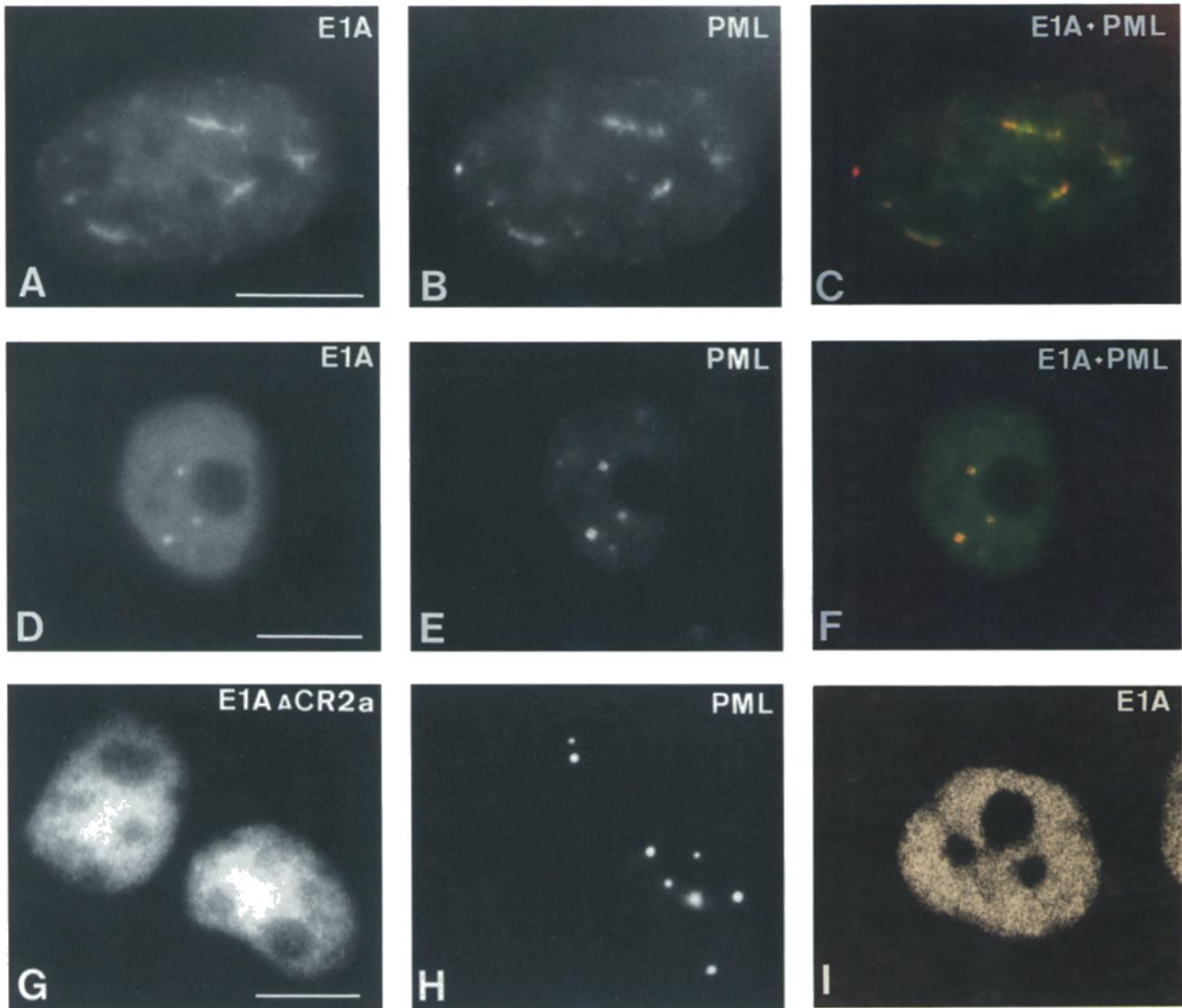


Figure 7. The E1A proteins colocalize with PML domains. (A–C) Indirect immunofluorescence was performed on HeLa cells at 14-h postinfection using the anti-E1A mAb M73 and PML serum. The cells were extracted with Triton X-100 and then fixed with paraformaldehyde. The binding sites of the mAb M73 were visualized with fluorescein (A) and those of PML antibodies with Texas red (B). (C; green staining for the E1A proteins, red staining for PML). Note that the yellow color is produced by overlapping of red and green staining). When infected cells were first fixed with paraformaldehyde and then treated with Triton X-100, the M73 antibody produced an intense diffuse staining of the nucleoplasm and concentration in PML domains was not clearly detected (I). (D–F) HeLa cells were transiently transfected with plasmid pE1A, which expresses E1A proteins, and double-labeled with M73 (D, visualized using fluorescein) and PML serum (E, visualized using Texas red). Images from each fluorochrome were recorded and superimposed (F, green staining for E1A proteins, red staining for PML). (G and H) HeLa cells were transiently transfected with plasmid p12SΔCR2a, which expresses an E1A-12S deletion mutant lacking amino acids 121–125. These cells were double-labeled with mAb M73 (G) and PML serum (H). Note that this deleted form of E1A does not concentrate in PML domains, even at high levels of E1A expression, as assessed by the intensity of the immunofluorescence signal (G). For D–H, that correspond to immunofluorescence performed in transfected cells, similar results were obtained when cells were permeabilized either before or after fixation. Bar, 10 μm.

The CR2 Region of the E1A Oncoproteins Is Necessary for the Association with the PML Nuclear Bodies

Having established that both E1A and E4-ORF3 encoded proteins colocalize with PML in adenovirus infected cells and that ORF3 is responsible for the redistribution of PML from nuclear bodies into nuclear fibrous structures, it was important to determine whether the apparent association between E1A and PML is restricted to these viral-

induced fibrous structures or whether it can also occur in the normal nuclear bodies. To address this question, HeLa cells were transiently transfected with vectors expressing E1A proteins and analyzed by indirect immunofluorescence using E1A and PML antibodies. The results show that the proteins expressed by transfection of the E1A gene are distributed throughout the nucleoplasm with additional concentrations in a few brighter dots that colocalize with the PML domains (Fig. 7, D–F). These foci were

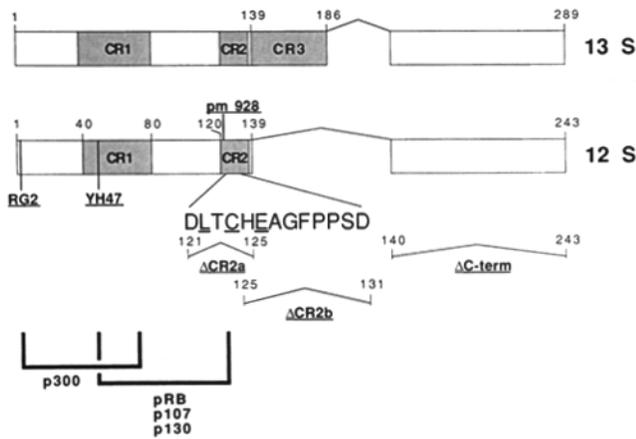


Figure 8. Schematic representation of the domain structure of E1A. Diagram of E1A proteins encoded by the 13S and 12S mRNAs indicating splice junctions, conserved regions 1, 2, and 3 (CR1-3), including the (D)LXCXE motif of CR2, and regions critical for p300, pRB, p107, and p130 binding (*upward lines*). The designations of specific mutations are underlined.

observed regardless of the method used (i. e., when cells were treated with Triton X-100 either before or after paraformaldehyde fixation).

To test whether the colocalization of E1A proteins with PML depends upon the sequences of E1A that are critical for efficient transactivation and/or transformation, we analyzed the intracellular localization of a series of E1A mutants (see Fig. 8, Table I). HeLa cells were first transfected with plasmids encoding either E1A-13S (which contains the CR1, CR2, and CR3 sequences) or E1A-12S (which only contains the CR1 and CR2 sequences). Both 12S and 13S proteins were detected diffusely distributed in the nucleoplasm with additional concentrations in the PML domains (Table I), indicating that the CR3 region is not required for the concentration of E1A in the PML bodies. Colocalization between E1A and PML was also observed in cells transfected with E1A mutants harboring deletions in either the CR1, the CR2b regions or in the carboxy-terminal exon (Table I). In contrast, E1A deletion mutants lacking both CR1 and CR2a regions or the CR2a region alone failed to concentrate in the PML domains (Fig. 7, *G* and *H*). This indicates that the (D)LXCXE motif which is disrupted in the Δ CR2a mutant (Fig. 8) appears critical for the concentration of E1A protein in the PML structures.

Since the integrity of this motif is required for the high affinity association of E1A to the pRB product and its related proteins p107 and p130 (reviewed by Moran, 1993; Bayley and Mymryk, 1994), we were interested in extending our analysis to other E1A mutants defective in binding these proteins. Cells were transfected with the following E1A point mutants: 12S.pm928 which abrogates pRB binding selectively, 12S.YH47 which abrogates both pRB and p130 binding, and the double point mutant 12S.YH47/pm928 which disrupts binding of pRB, p107, and p130 proteins (Wang et al., 1993). None of these mutations affected the normal colocalization of E1A with PML (Table I). We therefore conclude that the sequences required for E1A concentration in PML domains overlap but are not identical to those involved in the interaction of E1A with pRB

Table I. Transfections with E1A and E1A Mutant DNAs and Co-immunolocalization with PML Protein

E1A Construct	E1A in PML Nuclear Bodies
pE1A	+
p13S	+
p12S	+
p12S.DCR1	+
p12S.DCR1,2a	-
p12S.DCR2a	-
p12S.DCR2b	+
p12S.DC-term*	+
p12S.pm 928	+
p12S.YH47	+
p12S.YH47/pm928	+
p12S.RG2	+

*E1A product detected with mAb M1; all others detected with mAb M73 (Harlow et al., 1985).

products. This is consistent with the finding that anti-pRB antibodies produce a diffuse staining of the nucleoplasm without any clear concentration in PML domains, both in uninfected and in adenovirus-infected cells, as well as in cells transfected with an pRB expression vector (data not shown).

In addition to the CR1 and CR2 regions, the NH₂ terminus of E1A products is also strictly required for E1A plus *ras* transformation and this function is believed to involve binding of another cellular protein, p300 (Subramanian et al., 1988; Whyte et al., 1989; Wang et al., 1993). In order to test whether this sequence is important for the colocalization of E1A with PML, HeLa cells were transiently transfected with the E1A point mutant 12S.RG2 which selectively abrogates p300 binding (Wang et al., 1993). Indirect immunofluorescence revealed that this E1A product retains the ability to concentrate within the PML domains (Table I). In this regard, antibodies directed against the nuclear p300 protein (Eckner et al., 1994) did not reveal any specific labeling of the PML domains but rather exhibited a homogeneous nuclear staining that excluded the nucleoli (data not shown).

The SV-40 Large T Antigen Accumulates in Close Proximity to PML Nuclear Bodies

As the (D)LXCXE motif present in the conserved region 2 of E1A appears to be required for the concentration of E1A proteins in the PML nuclear bodies, we decided to examine whether other viral gene products which contain similar amino acid motifs can also interact with these domains. To address this question, cells were transiently transfected with a vector expressing the early SV-40 proteins (Banerji et al., 1983) and analyzed by immunofluorescence using an antibody specific to the Large T antigen (LT). The results show that LT is distributed throughout the nucleoplasm and, in addition, concentrates in discrete foci (Fig. 9 A). These foci are particularly evident when the cells are mildly extracted with Triton X-100 prior to formaldehyde fixation. Double-labeling experiments using LT and PML antibodies reveal that the sites of LT concentration are frequently associated with the PML nuclear bodies (Fig. 9, A-C). In a total of 20 transfected cells, we observed that 83% of the foci labeled by LT antibody are

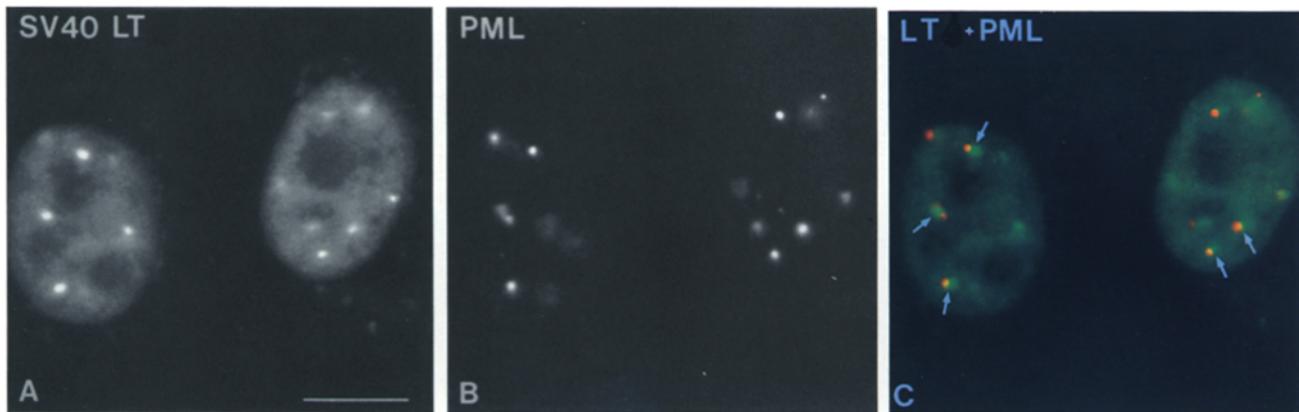


Figure 9. SV-40 large T antigen associates with the PML domains. HeLa cells were transiently transfected with plasmid pDept which encodes early SV-40 proteins (Banerji et al., 1983). The cells were permeabilized with Triton X-100 and then fixed with paraformaldehyde. The large T antigen (LT) was detected using mAb Ab-1 and visualized with fluorescein (A). PML was visualized using rabbit antiserum and Texas Red (B). C represents the superimposition of confocal images obtained from each fluorochrome (green staining for LT, red staining for PML). Note that the structures labeled by antibodies directed against LT and PML are closely associated but do not colocalize precisely (arrows). Bar, 10 μ m.

in close proximity to PML nuclear bodies. These results indicate that, in transfected cells, in addition to being diffusely distributed in the nucleoplasm, LT concentrates within distinct subnuclear domains, a high percentage of which associate with the PML nuclear bodies.

Discussion

Here we report that infection by adenovirus causes a drastic redistribution of PML from spherical to fibrous-appearing nuclear structures, which, at the electron microscopic level, consist of a meshwork of filaments of approximately 12 nm in diameter. A distinct component of the normal PML nuclear bodies, the SP100 protein (Szostecki et al., 1990; Xie et al., 1993), is also detected in the adenovirus-induced fibrous structures in a subset of infected cells. Although we cannot exclude that the disappearance of SP100 staining could be due to a dispersion of the protein, it is more likely that SP100 is also present in the fibers, but is more difficult to detect, perhaps because it becomes less accessible for antibody binding. This argues that adenovirus does not only affect the specific localization of PML, but rather disorganizes the whole macromolecular complex into fibrillar structures. A modification of the PML nuclear domain staining pattern was also observed in Herpes Simplex Virus (HSV)-infected cells (Maul et al., 1993). However, in this case, viral infection completely abolished the labeling by antibodies directed against components of these nuclear domains, suggesting a complete disaggregation of the complexes. These findings indicate the existence of an intimate association between the modification of the PML nuclear bodies and events surrounding infection by different types of DNA viruses.

Using a series of adenovirus early region 4 (E4) mutant viruses and plasmid vectors expressing single E4-ORFs, we demonstrate that the ORF3 product is responsible for the disorganization of the PML subnuclear structures. In this context, it is noteworthy that both PML (Stuurman et al., 1992) and the 11-kD protein encoded by E4-ORF3 (Sarnow et al., 1982) have been shown to be tightly associ-

ated with the nuclear matrix, the structure and function of which is still poorly understood. The elucidation of the biological properties of E4-ORF3 should contribute to our understanding of nuclear matrix function. The adenovirus E4 region is a complex transcription unit that encodes at least seven distinct proteins (Freyer et al., 1984; Virtanen et al., 1984) and specific biological activities have only been attributed to four of them. The ORF4 product is an activator of protein phosphatase 2A (Kleinberger and Shenk, 1993). The ORF6/7 hybrid protein activates transcription from the adenovirus E2 promoter by facilitating cooperative DNA-binding of E2F-1/DP-1 heterodimers (Helin and Harlow, 1994; Bandara et al., 1994 and references therein). The ORF6 protein forms a complex with the E1B 55K protein that inhibits cytoplasmic accumulation of host cell mRNA and facilitates transport of late virus-encoded mRNAs (Cutt et al., 1987; Bridge and Ketner, 1990). In addition, ORF 3 and 6 proteins have been shown to be jointly implicated in the regulation of alternative splicing of viral mRNAs (Öhman et al., 1993; Nordqvist et al., 1994) and viral DNA synthesis (Huang and Hearing, 1989; Bridge and Ketner, 1989). These two products have redundant activities during lytic infection, since expression of one of them is sufficient to substitute for the whole E4 region in establishing a wild type virus infection (Huang and Hearing, 1989; Bridge and Ketner, 1989).

Our finding that the ORF3 protein is alone sufficient for causing the redistribution of PML implies that the reorganization of the associated nuclear bodies is not essential for viral lytic infections since the ORF6 product can functionally replace ORF3 in virus production without concomitant PML redistribution. The biological significance of the ORF3-induced reorganization of the PML bodies remains unclear. The only data reported to date on the functions of ORFs 3 and 6 suggest that they may be involved in RNA splicing, since ORF3 was shown to facilitate exon inclusion and ORF6 exon skipping of major late mRNAs (Nordqvist et al., 1994). In this respect, it is noteworthy that snRNPs have recently been shown to reorganize upon adenovirus infection (Puvion-Dutilleul et al.,

1994). If the PML-containing nuclear fibers were to represent active sites of mRNA processing, one might have expected a colocalization of ORF6 with ORF3 in such structures. In fact, the distribution of ORF6 was found to be exclusively nuclear diffuse. Moreover, previous experiments on non-infected cells have failed to demonstrate any colocalization between the intact PML nuclear bodies and snRNP-containing organelles (Weis et al., 1994), nor do we show here any correlation between the ORF3-induced fibers and sites of RNA splicing. The PML nuclear bodies may therefore represent active sites for an additional ORF3 function, distinct from its possible role in RNA splicing.

Because the disruption of the PML-associated nuclear structures was first implicated in an oncogenic process (Weis et al., 1994; Dyck et al., 1994; Koken et al., 1994), we were interested in examining the intracellular localization of the adenoviral E1A oncoproteins. In addition to being diffusely distributed in the nucleoplasm, a significant portion of the E1A proteins concentrated in the PML nuclear structures. Further, the E1A products were seen both in the abnormal, ORF3-induced fibers resulting from infection, as well as in the normal spherical bodies seen upon transfection of either the 12S or 13S E1A cDNAs. The biological significance for this concentration remains to be elucidated. The spatial proximity of E1A and PML in subnuclear structures is unlikely to be the result of direct association since both co-immunoprecipitation and *in vitro* binding studies failed to detect any specific interaction between the two proteins (our unpublished results). Unexpectedly, E1A does not accumulate in the otherwise intact nuclear bodies of 293 cells (which constitutively express E1A), but rather exhibits an exclusively diffuse distribution within the nucleoplasm. However, upon transfection of an E1A expression plasmid, E1A was now found to concentrate into the PML structures (our unpublished observations). It remains to be established whether the PML nuclear bodies simply function as storage organelles for the overexpression-dependent accumulation of E1A, or whether they represent subnuclear compartments for specific E1A-associated biochemical processes.

Distinct biological functions have been attributed to discrete amino acid sequences in adenovirus E1A proteins. Identification of the region responsible for the concentration of E1A in the PML nuclear bodies might therefore provide clues as to the function of the whole macromolecular complex. Analysis of the nuclear localization of mutant E1A proteins revealed that the (D)LXCXE motif in the conserved region 2 (CR2) is critical for the association of E1A with the PML nuclear bodies. This motif, which is also present in other DNA tumor virus oncoproteins, including large T antigens from simian virus 40 (SV-40 LT) and polyomavirus as well as the human papillomavirus E7 protein, has been shown to be essential for binding to pRB and to the related p107 and p130 proteins (reviewed in Moran, 1993, and Bayley and Mymryk, 1994). In E1A, a single substitution within this motif, changing Cys-124 to Gly (pm 928), has been shown to abrogate both pRB binding and E1A transforming ability. Since the pm928 mutation did not affect E1A accumulation in the PML bodies, we suggest that the targeting of E1A to these structures is dependent on a CR2 function distinct from its pRB bind-

ing properties. In this regard, we failed to demonstrate any concentration of the endogenous pRB in the PML substructures. However, when overexpressed together with PML in transfected cells, pRB accumulated into large nuclear aggregates which we did not investigate further (our unpublished observations). Finally, the SV-40 LT antigen was also shown to concentrate in discrete domains closely associated with those containing PML. Taken together, our results demonstrate that the nuclear matrix-associated PML structures represent preferential targets for small DNA tumor virus oncoproteins. Further analysis of the composition and function of the PML nuclear bodies should help elucidate the biological implications of the present observations.

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